



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US96/13363  <b>(22) International Filing Date:</b> 16 August 1996 (16.08.96)  <b>(30) Priority Data:</b> 60/002,490 18 August 1995 (18.08.95) US 60/002,479 18 August 1995 (18.08.95) US  <b>(71) Applicant (for all designated States except US):</b> SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ROTHMAN, James, E. [US/US]; Apartment 10B, 402 East 64th Street, New York, NY 10021 (US). HARTL, F., Ulrich [DE/US]; 20 Quarry Lane, Irvington, NY 10533 (US). HOE, Mee, H. [MY/US]; 312 East 66th Street, New York, NY 10021 (US). HOUGHTON, Alan [US/US]; Apartment 7C, 402 East 64th Street, New York, NY 10021 (US). TAKEUCHI, Yoshizumi [JP/US]; Apartment 24M, 1635 York Avenue, New York, NY 10021 (US). MAYHEW, Mark [GB/US]; Apartment 16A, 10 River Road, New York, NY 10044 (US).		<b>(74) Agents:</b> LARSON, Marina et al.; Oppedahl & Larson, Suite 309, 1992 Commerce Street, Yorktown Heights, NY 10598 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> HEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES  <b>(57) Abstract</b>  <p>The present invention relates to methods and compositions for inducing an immune response in a subject, wherein the subject is administered an effective amount of at least one heat shock protein in combination with one or more defined target antigens. These methods and compositions may be used in the treatment of infectious diseases and cancers.</p>		

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DescriptionHEAT SHOCK PROTEIN-BASED VACCINES AND IMMUNOTHERAPIES

The invention described herein was made in the course of work under NIH Core Grant No. CA 08748. The United States government may have certain rights in this invention.

5

Introduction

The present invention relates to methods and compositions for inducing an immune response in a subject, wherein the subject is administered an effective amount of at least one heat shock protein in combination with one or more defined target antigens. These methods and compositions may be used in the treatment of infectious diseases and cancers.

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Background of the Invention

Heat shock proteins were originally observed to be expressed in increased amounts in mammalian cells which were exposed to sudden elevations of temperature, while the expression of most cellular proteins is significantly reduced. It has since been determined that such proteins are produced in response to various types of stress, including glucose deprivation. As used herein, the term "heat shock protein" will be used to encompass both proteins that are expressly labeled as such as well as other stress proteins, including homologs of such proteins that are expressed constitutively (i.e., in the absence of stressful conditions). Examples of heat shock proteins include BiP (also referred to as grp78), hsp/hsc70, gp96 (grp94), hsp60, hsp40 and hsp90.

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Heat shock proteins have the ability to bind other proteins in their non-native states, and in particular to bind nascent peptides emerging from ribosomes or extruded into the endoplasmic reticulum. Hendrick and Hartl., Ann. Rev. Biochem. 62:349-384 (1993); Hartl., Nature 381:571-580 (1996). Further, heat shock proteins have been shown to play

an important role in the proper folding and assembly of proteins in the cytosol, endoplasmic reticulum and mitochondria; in view of this function, they are referred to as "molecular chaperones". Frydman et al., *Nature* 370: 111-117 (1994); Hendrick and Hartl., *Ann. Rev. Biochem.* 62:349-384 (1993); Hartl, *Nature* 381:571-580 (1996).

For example, the protein BiP, a member of a class of heat shock proteins referred to as the hsp70 family, has been found to bind to newly synthesized, unfolded  $\mu$  immunoglobulin heavy chain prior to its assembly with light chain in the endoplasmic reticulum. Hendershot et al., *J. Cell Biol.* 104:761-767 (1987). Another heat shock protein, gp96, is a member of the hsp90 family of stress proteins which localize in the endoplasmic reticulum. Li and Srivastava, *EMBO J.* 12:3143-3151 (1993); Mazzarella and Green, *J. Biol. Chem.* 262:8875-8883 (1987). It has been proposed that gp96 may assist in the assembly of multi-subunit proteins in the endoplasmic reticulum. Wiech et al., *Nature* 358:169-170 (1992).

It has been observed that heat shock proteins prepared from tumors in experimental animals were able to induce immune responses in a tumor-specific manner; that is to say, heat shock protein purified from a particular tumor could induce an immune response in an experimental animal which would inhibit the growth of the same tumor, but not other tumors. Srivastava and Maki, 1991, *Curr. Topics Microbiol.* 167:109-123 (1991). The source of the tumor-specific immunogenicity has not been confirmed. Genes encoding heat shock proteins have not been found to exhibit tumor-specific DNA polymorphism. Srivastava and Udonio, *Curr. Opin. Immunol.* 6:728-732 (1994). High resolution gel electrophoresis has indicated that gp96 may be heterogeneous at the molecular level. Feldweg and Srivastava, *Int. J. Cancer* 63: 310-314 (1995). Evidence suggests that the source of heterogeneity may be populations of small peptides adherent to the heat shock protein, which may number in the hundreds. *Id.* It has been proposed that a wide diversity of peptides

adherent to tumor-synthesized heat shock proteins may render such proteins capable of eliciting an immune response in subjects having diverse HLA phenotypes, in contrast to more traditional immunogens which may be somewhat HLA-restricted in their efficacy. *Id.*

Recently, Nieland et al. (*Proc. Natl. Acad. Sci. U.S.A.* 93:6135-6139 (1996)) identified an antigenic peptide containing a cytotoxic T lymphocyte (CTL) vesicular stomatitis virus (VSV) epitope bound to gp96 produced by VSV-infected cells. Nieland's methods precluded the identification of any additional peptides or other compounds which may also have bound to gp96, and were therefore unable to further characterize higher molecular weight material which was bound to gp96 and detected by high pressure liquid chromatography.

It has been reported that a synthetic peptide comprising multiple iterations of NANP (Asp Ala Asp Pro) malarial antigen, chemically cross-linked to glutaraldehyde-fixed mycobacterial hsp65 or hsp70, was capable of inducing antibody formation (i.e., a humoral response) in mice in the absence of any added adjuvant; a similar effect was observed using heat shock protein from the bacterium *Escherichia coli*. Del Guidice, *Experientia* 50:1061-1066 (1994); Barrios et al., *Clin. Exp. Immunol.* 98:224-228 (1994); Barrios et al., *Eur. J. Immunol.* 22:1365-1372 (1992). Cross-linking of synthetic peptide to heat shock protein and possibly glutaraldehyde fixation was required for antibody induction. Barrios et al., *Clin. Exp. Immunol.* 98:229-233.

It has now been discovered, according to the present invention, that heat shock protein may be combined with target antigen and used to induce an immune response which includes a cytotoxic cellular component, i.e., a cellular response.

#### Summary of the Invention

The present invention relates to methods and compositions for inducing an immune response in a subject, wherein

at least one heat shock protein in combination with one or more defined target antigens is administered to the subject.

Unlike prior disclosures relating to heat shock protein associated with an undefined population of potential  
5 antigens which have been restricted, in their immunogenic effect, to a single tumor, the present invention provides for methods and compositions which combine heat shock protein with a defined target antigen which may be selected  
10 on the basis that it is immunogenic in diverse occurrences of a neoplastic or infectious disease, or because it has been identified, in an individual instance, as being particularly immunogenic. Further, because the use of one or more defined target antigen permits more control over the immune response elicited, it may avoid the induction of an  
15 undesirable immune response.

In alternative embodiments of the invention, the target antigen may be either (i) an antigen which itself binds to the heat shock protein; or (ii) a hybrid antigen comprising an immunogenic domain as well as a heat shock  
20 protein-binding domain. The immunogenic domain may be an entire protein or peptide antigen, or may be only a portion of the selected antigen, for example a selected epitope of the antigen. In specific, nonlimiting embodiments of the invention, the heat shock protein binding domain may  
25 comprise a peptide having the sequence:

His Trp Asp Phe Ala Trp Pro Trp [SEQ. ID NO. 1]

The present invention provides for methods of administering such heat shock protein/target antigen compositions comprising (i) combining one or more heat shock  
30 protein with one or more target antigens in vitro, under conditions wherein binding of target antigen to heat shock protein occurs to form a target antigen/heat shock protein complex; and (ii) administering the target antigen, bound to heat shock protein, in an effective amount to a subject in  
35 need of such treatment.

Alternatively, heat shock protein/target antigen combinations of the invention may be administered to a

subject by introducing nucleic acid encoding the heat shock protein and the target antigen into the subject such that the heat shock protein and target antigen bind *in situ*.

#### Brief Description of the Drawings

5 Fig. 1 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

10 Fig. 2 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

Fig. 3 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

15 Fig. 4 shows the induction of a cellular immune response using hybrid peptide antigens in accordance with the invention;

20 Figs. 5A and 5B shows the results of control experiments in which hybrid peptide or Ova-peptide and heat shock protein were administered individually to EL4 cells;

Fig. 6 shows co-elution of hybrid peptides and heat shock proteins from a column, demonstrating binding of the polypeptides to the heat shock protein;

Fig. 7 shows the co-elution of  $^{125}\text{I}$ -OVA-BiP with BiP in the presence and absence of ATP;

25 Fig. 8 shows the killing efficacy of T-cells primed with various combinations of antigens and heat shock proteins on EL4 cells pulsed with antigen; and

30 Fig. 9 shows the killing efficacy of T-cells primed with various concentrations of antigens and heat shock proteins on EG7 lymphoma cells.

#### Detailed Description of the Invention

For purposes of clarity of description, and not by way of limitation, the detailed description is divided into the following subsections:

35 (i) heat shock proteins;

- (ii) target antigens; and
- (iii) methods of administration.

### Heat Shock Proteins

The term "heat shock protein", as used herein,  
5 refers to any protein which exhibits increased expression in  
a cell when the cell is subjected to a stress. In preferred  
nonlimiting embodiments, the heat shock protein is  
originally derived from a eukaryotic cell; in more preferred  
embodiments, the heat shock protein is originally derived  
10 from a mammalian cell. For example, but not by way of  
limitation, heat shock proteins which may be used according  
to the invention include BiP (also referred to as grp78),  
hsp/hsc70, gp96 (grp94), hsp60, hsp40, and hsp90. Especially  
preferred heat shock proteins are BiP, gp96, and hsp70, as  
15 exemplified below. Naturally occurring or recombinantly  
derived mutants of heat shock proteins may also be used  
according to the invention. For example, but not by way of  
limitation, the present invention provides for the use of  
heat shock proteins mutated so as to facilitate their  
20 secretion from the cell (for example having mutation or  
deletion of an element which facilitates endoplasmic  
reticulum recapture, such as KDEL or its homologs; such  
mutants are described in concurrently filed PCT Application  
No. \_\_\_\_\_ (Attorney Docket No. MSK.P-018), which is  
25 incorporated herein by reference.

For embodiments of the invention wherein heat shock  
protein and target antigen are directly administered to the  
subject in the form of a protein/peptide complex, the heat  
shock protein may be prepared, using standard techniques,  
30 from natural sources, for example as described in Flynn et  
al., *Science* 245: 385-390 (1989), or using recombinant tech-  
niques such as expression of a heat shock encoding vector in  
a suitable host cell such as a bacterial, yeast or mammalian  
cell. If pre-loading of the heat shock protein with  
35 peptides from the host organism is a concern, the heat shock  
protein can be incubated with ATP and then repurified.



Nonlimiting examples of methods for preparing recombinant heat shock proteins are set forth below.

A nucleic acid encoding a heat shock protein may be operatively linked to elements necessary or desirable for expression and then used to express the desired heat shock protein as either a means to produce heat shock protein for use in a protein vaccine or, alternatively, in a nucleic acid vaccine. Elements necessary or desirable for expression include, but are not limited to, promoter/enhancer elements, transcriptional start and stop sequences, polyadenylation signals, translational start and stop sequences, ribosome binding sites, signal sequences and the like. For example, but not by way of limitation, genes for various heat shock proteins have been cloned and sequenced, including, but not limited to, gp96, human: Genebank Accession No. X15187; Maki et al., *Proc. Nat'l Acad. Sci.* 87: 5658-5562 (1990), mouse: Genebank Accession No. M16370; Srivastava et al., *Proc. Nat'l Acad. Sci.* 84:3807-3811 (1987)); BiP, mouse: Genebank Accession No. U16277; Haas et al., *Proc. Nat'l Acad. Sci. U.S.A.* 85: 2250-2254 (1988), human: Genebank Accession No. M19645; Ting et al., *DNA* 7: 275-286 (1988); hsp70, mouse: Genebank Accession No. M35021; Hunt et al., *Gene* 87: 199-204 (1990), human: Genebank Accession No. M24743; Hunt et al., *Proc. Nat'l Acad. Sci. U.S.A.* 82: 6455-6489 (1995); and hsp40 human: Genebank Accession No. D49547; Ohtsuka K., *Biochem Biophys. Res. Commun.* 197: 235-240 (1993).

#### Target Antigens

A target antigen, according to the invention, may be either (i) an antigen which itself binds to the heat shock protein; or (ii) a hybrid antigen comprising an immunogenic domain as well as a heat shock protein-binding domain. Thus, the target antigen serves at least two functions, namely (I) it contains an epitope capable of inducing the desired immune response; and (ii) it is capable of physically binding to its partner heat shock protein. Of note, the term "physically binds" indicates that the target antigen and

heat shock protein exhibit a physical interaction which permits the adherence of one to the other for at least a transient period of time; of note, the binding need not, and in most embodiments of the invention should not, be  
5 irreversible.

In certain embodiments, an antigen capable of inducing the desired immune response may be found to be inherently capable of binding to a partner heat shock protein. In other embodiments, it may be necessary or desirable to link an immunogenic antigen to one or more other  
10 compounds so as to create a hybrid antigen which contains both an immunogenic domain as well as a heat shock protein binding domain. In such circumstances, a compound which is, itself, an immunogenic antigen may be linked to a compound  
15 which is, itself, capable of binding to a heat shock protein. Alternatively, the linkage of two or more compounds which individually lack either functionality may give rise to the desired immunogenic and binding characteristics.

The term "antigen" as used herein, refers to a  
20 compound which may be composed of amino acids, carbohydrates, nucleic acids or lipids individually or in any combination.

The term "target antigen", as used herein, refers to a compound which binds to one or more heat shock proteins  
25 and which is representative of the immunogen toward which an immune response is desirably directed. For example, where the immunogen is an influenza virus, the target antigen may be a peptide fragment of the matrix protein of the influenza virus. As used herein, the term "immunogen" is applied to  
30 the neoplastic cell, infected cell, pathogen, or component thereof, towards which an immune response is to be elicited, whereas the target antigen is a portion of that immunogen which can provoke the desired response and which inherently or through engineering binds to one or more heat shock  
35 proteins. In particular, the target antigen is selected to elicit an immune response to a particular disease or pathogen, including peptides obtained from MHC molecules,

mutated DNA gene products, and direct DNA products such as those obtained from tumor cells.

While the invention may be applied to any type of immunogen, immunogens of particular interest are those associated with, derived from, or predicted to be associated with a neoplastic disease, including but not limited to a sarcoma, a lymphoma, a leukemia, or a carcinoma, and in particular, with melanoma, carcinoma of the breast, carcinoma of the prostate, ovarian carcinoma, carcinoma of the cervix, colon carcinoma, carcinoma of the lung, glioblastoma, astrocytoma, etc. Further, mutations of tumor suppressor gene products such as p53, or oncogene products such as ras may also provide target antigens to be used according to the invention.

In further embodiments, the immunogen may be associated with an infectious disease, and, as such, may be a bacterium, virus, protozoan, mycoplasma, fungus, yeast, parasite, or prion. For example, but not by way of limitation, the immunogen may be a human papilloma virus (see below), a herpes virus such as herpes simplex or herpes zoster, a retrovirus such as human immunodeficiency virus 1 or 2, a hepatitis virus, an influenza virus, a rhinovirus, respiratory syncytial virus, cytomegalovirus, adenovirus, *Mycoplasma pneumoniae*, a bacterium of the genus *Salmonella*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Escherichia*, *Klebsiella*, *Vibrio*, *Mycobacterium*, amoeba, a malarial parasite, *Trypanosoma cruzi*, etc.

Immunogens may be obtained by isolation directly from a neoplasm, an infected cell, a specimen from an infected subject, a cell culture, or an organism culture, or may be synthesized by chemical or recombinant techniques. Suitable antigenic peptides, particularly for use in a hybrid antigen, for use against viruses, bacteria and the like can be designed by searching through their sequences for MHC class I restricted peptide epitopes containing HLA binding sequences such as but not limited to HLA-A2 peptide binding sequences:

Xaa (Leu/Met) XaaXaaXaa (Val/Ile/Leu/Thr) XaaXaa (Val/Leu)

[SEQ ID No. 2],

Rammensee et al., *Immunogenetics* 41: 178-223 (1995),

Xaa (Leu/Met) XaaXaaXaaXaaXaaXaaVal

[SEQ ID No. 3],

5 Tarpey, et al *Immunology* 81: 222-227 (1994),

Xaa (Val/Gln) XaaXaaXaaXaaXaaXaaLeu

[SEQ ID No. 28]

Barouch et al., *J. Exp. Med.* 182: 1847-1856 (1995).

10 It may also be desirable to consider the type of  
immune response which is desired. For example, under certain  
circumstances, a humoral immune response may be appropriate.  
In other cases, and indeed where an immune response directed  
toward neoplastic cells or infected cells is sought to be  
elicited, a cellular immune response is particularly desir-  
able. Accordingly, particular epitopes associated with the  
15 activation of B cells, T helper cells, or cytotoxic T cells  
may be identified and selected for incorporation into the  
target antigen.

20 It may also be desirable to utilize target antigen  
associated with an autoimmune disease or allergy. Such a  
target antigen may be administered, together with one or  
more heat shock proteins, in an amount sufficient to be  
tolerogenic or to inhibit a pre-existing immune response to  
the target antigen in a subject. The amount of heat shock  
protein required to inhibit the immune response is expected  
25 to be substantially greater than the amount required for  
stimulation.

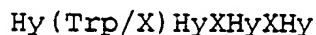
30 Although the size of target antigen may vary  
depending upon the heat shock protein used, in nonlimiting  
embodiments of the invention, the target antigen may be the  
size of a peptide having between 4 and 500 of amino acid  
residues, and preferably be the size of a peptide having  
between 4 and 100, most preferably 7 and 20 amino acid  
residues. As such, it may be desirable to produce a fragment  
of an immunogen to serve as a target antigen, or, alterna-  
35 tively, to synthesize a target antigen by chemical or  
recombinant DNA methods. In some instances, however, an  
immunogen may, in intact form, serve as a target antigen.

Based on the foregoing considerations, a target antigen may be prepared, and then tested for its ability to bind to heat shock protein. In some instances, binding of target antigen to a particular heat shock protein may be facilitated by the presence of at least one other protein, which may be a heat shock protein.

For example, binding of target antigen to a heat shock protein may be evaluated by labeling the target antigen with a detectable label, such as a radioactive, fluorescent, enzymatic or pigmented label, combining the target antigen with heat shock protein under conditions which would be expected to permit binding to occur, and then isolating the heat shock protein while removing any unbound target antigen, and determining whether any labeled target antigen had adhered to the heat shock protein. As a specific example, and not by way of limitation, the ability of a target antigen to bind to BiP heat shock protein may be evaluated by combining 2  $\mu$ g BiP with up to about 1150 pmole of radioactively labeled target antigen in buffer containing 50 mM Tris, HCl (pH 7.5), 200 mM NaCl, and 1 mM Na<sub>2</sub>EDTA, in a final volume of 50  $\mu$ l, for 30 minutes at 37 degrees Centigrade. Unbound target antigen may then be removed from bound BiP-target antigen by centrifugation at 100g by desalting through a 1 ml Sephadex-G column for 2 minutes. Penefsky, J. Biol. Chem. 252:2891 (1977). To prevent binding to the resin, columns may first be treated with 100  $\mu$ l of bovine serum albumin in the same buffer and centrifuged as above. Bound target antigen may then be quantitated by liquid scintillation counting. See Flynn et al., Science 245:385-390 (1989).

Because ATP hydrolysis drives the release of peptides from many known heat shock proteins, the amount of ATPase activity may often be used to quantitate the amount of target antigen binding to heat shock protein. An example of how such an assay may be performed is set forth in Flynn et al., Science 245:385-390 (1990).

If a particular immunogen or a fragment thereof does not satisfactorily bind to a heat shock protein, then that immunogen or fragment may be linked to another compound so as to create a heat shock protein-binding domain thereby constructing a hybrid antigen. The heat shock protein-binding domain is selected so that the hybrid peptide will bind in vitro to a heat shock protein such as BiP, hsp70, gp96, or hsp90, alone or in combination with accessory heat shock proteins such as hsp40, or hsp60. Peptides which fulfill this criterion may be identified by panning libraries of antigens known to bind well to one or more heat shock proteins as described in Blond-Elguindi et al., *Cell* 75:717-728 (1993). Using this technique, Blond-Elguindi have concluded that the heat shock protein BiP recognizes polypeptides that contain a heptameric region having the sequence



where Hy represents a hydrophobic amino acid residue, particularly tryptophan, leucine or phenylalanine, and X is any amino acid. High affinity heat-shock protein-binding sequences incorporating this motif include:

His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1]; and  
Phe Trp Gly Leu Trp Pro Trp Glu [Seq. ID No. 4].

Other heat shock protein binding motifs have also been identified. For example, Auger et al. *Nature Medicine* 2:306-310 (1996) have identified two pentapeptide binding motifs

Gln Lys Arg Ala Ala [SEQ ID No. 5] and

Arg Arg Arg Ala Ala [Seq. ID No. 6]

in HLA-DR types associated with rheumatoid arthritis which bind to heat shock proteins. Heat shock binding motifs have also been identified as consisting of seven to fifteen residue long peptides which are enriched in hydrophobic amino acids. Flynn et al., *Science* 245: 385-390 (1989); Gragerov et al., *J. Molec. Biol.* 235: 848-854 (1994).

The hybrid antigen of the invention incorporates one immunogenic domain and one heat shock protein-binding

domain, optionally separated by a short peptide linker. The hybrid peptide of the invention may be synthesized using chemical peptide synthesis methods or it can be synthesized by expression of a nucleic acid construct containing linked sequences encoding the antigenic and heat shock protein-binding domains. One suitable technique utilizes initial separate PCR amplification reactions to produce separate DNA segments encoding the two domains, each with a linker segment attached to one end, followed by fusion of the two amplified products in a further PCR step. This technique is referred to as linker tailing. Suitable restriction sites may also be engineered into regions of interest, after which restriction digestion and ligation is used to produce the desired hybrid peptide-encoding sequence.

#### Methods of Administration

The heat shock protein/target antigen combinations of the invention may be administered to a subject using either a protein-based or nucleic acid vaccine, so as to produce, in the subject, an amount of heat shock protein/target antigen complex which is effective in inducing a therapeutic immune response in the subject.

The subject may be a human or nonhuman subject.

The term "therapeutic immune response", as used herein, refers to an increase in humoral and/or cellular immunity, as measured by standard techniques, which is directed toward the target antigen. Preferably, but not by way of limitation, the induced level of humoral immunity directed toward target antigen is at least four-fold, and preferably at least 16-fold greater than the levels of the humoral immunity directed toward target antigen prior to the administration of the compositions of this invention to the subject. The immune response may also be measured qualitatively, wherein by means of a suitable *in vitro* assay or *in vivo* an arrest in progression or a remission of neoplastic or infectious disease in the subject is considered to indicate the induction of a therapeutic immune response.

Specific amounts of heat shock protein/target antigen administered may depend on numerous factors including the immunogenicity of the particular vaccine composition, the immunocompetence of the subject, the size of the subject and the route of administration. Determining a suitable amount of any given composition for administration is a matter of routine screening.

In specific nonlimiting embodiments of the invention, it may be desirable to include more than one species of heat shock protein, and/or more than one target antigen, in order to optimize the immune response. Such an approach may be particularly advantageous in the treatment of cancer or in the treatment of infections characterized by the rapid development of mutations that result in evasion of the immune response.

In other specific nonlimiting embodiments of the invention, in order to promote binding among members of each heat shock protein/target antigen pair, the ratio of heat shock protein to target antigen may preferably be 1:2 to 1:200. Higher relative levels of antigen are suitable to enhance binding to the heat shock protein.

According to still further specific but nonlimiting embodiments of the invention, the target antigen is not chemically cross-linked to the heat shock protein.

Compositions comprising target antigen/heat shock protein as set forth above are referred to herein as "vaccines". The term vaccine is used to indicate that the compositions of the invention may be used to induce a therapeutic immune response.

A vaccine composition comprising one or more heat shock proteins and one or more target antigens in accordance with the invention may be administered cutaneously, subcutaneously, intravenously, intramuscularly, parenterally, intrapulmonarily, intravaginally, intrarectally, nasally or topically. The vaccine composition may be delivered by injection, particle bombardment, orally or by aerosol.



Incubation of heat shock proteins in solution with the target antigen is sufficient to achieve loading of the antigen onto the heat shock protein in most cases. It may be desirable in some cases, however, to add agents which can assist in the loading of the antigen.

Incubation with heating of the heat shock protein with the target antigen will in general lead to loading of the antigen onto the heat shock protein. In some cases, however, it may be desirable to add additional agents to assist in the loading. For example, hsp40 can facilitate loading of peptides onto hsp70. Minami et al., *Gen. Biol Chem.* 271: 19617-19624 (1996). Denaturants such as guanidinium HCl or urea can be employed to partially and reversibly destabilize the heat shock protein to make the peptide binding pocket more accessible to the antigen.

Vaccine compositions in accordance with the invention may further include various additional materials, such as a pharmaceutically acceptable carrier. Suitable carriers include any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

The vaccine composition of the invention may also include suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions may be in the form of liquid or lyophilized or otherwise dried formulations and may include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and

ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g. glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexing with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc. or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. The choice of compositions will depend on the physical and chemical properties of the vaccine. For example, a product derived from a membrane-bound form of a protein may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including intramuscular, parenteral, pulmonary, nasal and oral.

As an alternative to direct administration of the heat shock protein and target antigen, one or more polynucleotide constructs may be administered which encode heat shock protein and target antigen in expressible form. The expressible polynucleotide constructs are introduced into cells in the subject using *ex vivo* or *in vivo* methods.

Suitable methods include injection directly into tissue and tumors, transfecting using liposomes (Fraley et al., *Nature* 370:111-117 (1980)), receptor-mediated endocytosis (Zatloukal, et al., *Ann. NY Acad. Sci.* 660:136-153 (1992));  
5 particle bombardment-mediated gene transfer (Eisenbraun et al., *DNA & Cell Biol.* 12:792-797 (1993)) and transfection using peptide presenting bacteriophage. Barry et al. *Nature Medicine* 2: 299-305 (1996). The polynucleotide vaccine may also be introduced into suitable cells *in vitro* which are  
10 then introduced into the subject.

To construct an expressible polynucleotide, a region encoding the heat shock protein and/or target antigen is prepared as discussed above and inserted into a mammalian expression vector operatively linked to a suitable promoter  
15 such as the SV40 promoter, the cytomegalovirus (CMV) promoter or the Rous sarcoma virus (RSV) promoter. The resulting construct may then be used as a vaccine for genetic immunization. The nucleic acid polymer(s) could also be cloned into a viral vector. Suitable vectors include but are not  
20 limited to retroviral vectors, adenovirus vectors, vaccinia virus vectors, pox virus vectors and adenovirus-associated vectors. Specific vectors which are suitable for use in the present invention are pCDNA3 (In-Vitrogen), plasmid AH5 (which contains the SV40 origin and the adenovirus major  
25 late promoter). pRC/CMV (InVitrogen), pCMU II (Paabo et al., *EMBO J.* 5:1921-1927 (1986)), pZip-Neo SV (Cepko et al., *Cell* 37:1053-1062 (1984)) and pSR $\alpha$  (DNAX, Palo Alto, CA).

#### EXAMPLE 1

##### PREPARATION OF HYBRID PEPTIDES

30 Hybrid peptides containing a BiP-binding domain (His-Trp-Asp-Phe-Ala-Trp-Pro-Trp; SEQ ID NO: 1) and an OVA antigenic domain (Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu; SEQ ID NO:7) separated by a tripeptide linker (gly-ser-gly) were synthesized. Peptides were produced in both orientations,  
35 OVA-BiP-binding domain and BiP-binding domain OVA as follows:

-18-

Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Gly-Ser-Gly-His-Trp-Asp-Phe-  
Ala-Trp-Pro-Trp [SEQ ID NO: 8]

and

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-Gly-Ser-Gly-Ser-Ile-Ile-Asn-  
5 Phe-Glu-Lys-Leu [SEQ ID NO. 9].

### EXAMPLE 2

Purified mouse cytosolic hsp70 was prepared from *E. coli* DH5 $\alpha$  cells transformed with pMS236 encoding mouse cytosolic hsp70. The cells were grown to an optical density  
10 (600 nm) of 0.6 at 37°C, and expression was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation 2 to 5 hours post induction and the pellets were resuspended to 20 mL with Buffer A (20 mM Hepes pH 7.0, 25 mM KCl, 1 mM DTT, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1mM  
15 PMSF). The cells were lysed by passing three times through a French press. The lysate was cleared by a low speed spin, followed by centrifugation at 100,000 X G for 30 minutes. The cleared lysate was applied to a Pharmacia XK26 column packed with 100 mL DEAE Sephacel and equilibrated with  
20 Buffer A at a flow rate of 0.6 cm/min. The column was washed to stable baseline with Buffer A and eluted with Buffer A adjusted to 175 mM KCl. The eluate was applied to a 25 mL ATP-agarose column (Sigma A2767), washed to baseline with Buffer A, and eluted with Buffer A containing 1 mM  
25 MgATP preadjusted to pH 7.0. EDTA was added to the eluate to a final concentration of 2 mM. The eluate which contained essentially pure hsp70 was precipitated by addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 80% saturation. The precipitate was resuspended in Buffer A containing 1 mM MgCl<sub>2</sub> and dialyzed against  
30 the same buffer with multiple changes. The purified hsp70 was frozen in small aliquots at -70°C.

### EXAMPLE 3

The purified hsp70 was combined with the synthesized peptides and used for immunization. To form the hsp70/  
35 peptide mixtures, approximately 15 ug (21.5 uM) hsp70 was

combined with 5 ug of Ova-peptide (0.5 mM, SEQ. ID. NO: 5) or 10 ug (0.5 mM) hybrid peptide (SEQ. ID NOS: 6 and 7) were mixed on ice to a final volume of 10  $\mu$ l in Buffer B (final concentration: 20 mM Hepes pH 7.0, 150 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub>, and 2 mM MgATP, pH 7.0). The mixtures were incubated for 30 minutes at 37°C and then used for *in vivo* immunizations.

C57BL/6 mice were immunized intradermally once a week for a total of two weeks with 10  $\mu$ L of one of the mixtures described above or with a mixture of TiterMax® (Vaxell, Norcross, GA) and Ova-peptide (5  $\mu$ g). One week after the second immunization, spleen cells were removed and mononuclear cells ( $6-8 \times 10^7$ ) were cultured with  $3 \times 10^6$   $\gamma$ -irradiated (3000 rad) stimulator cells. The stimulator cells were obtained from naive mice that had been sensitized *in vitro* with Ova-peptide (10 mg/ml) for 30 minutes at room temperature, washed and irradiated at 3000 rads.

Cytotoxicity of spleen cells from vaccinated mice were assayed on Ova-peptide pulsed EL4 cells in an 18-hour chromium release assay. CTL were generated by culturing *in vivo* immunized spleen cells for 5 days at a concentration of  $10^6$  cells/mL in RPMI medium, 10% FCS, penicillin, streptomycin and 2 mM L-glutamine, together with  $3 \times 10^6$   $\gamma$ -irradiated (3,000 rad) stimulator cells/mL. Target cells were prepared by culturing cells for 1 hour in the presence of 250  $\mu$ Ci of <sup>51</sup>Cr sodium chromate (DuPont, Boston, MA) in Tris-phosphate buffer, pH 7.4 at 37°C for 60 minutes. After washing,  $10^4$  <sup>51</sup>Cr-labeled target cells were mixed with effector lymphocytes to yield several different effector/target (E/T) ratios and were incubated for 18 hours. Supernatants were harvested and the radioactivity was measured in a gamma counter. Percent specific lysis was calculated as:  $100 \times [(\text{cpm release by CTL} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release})]$ . Maximal response was determined by addition of 1% Triton X-100. Spontaneous release of all target in the absence of effector cells was less than 25% of the maximal release.

As shown in Figure 1, the combination of Hsp70 and the hybrid peptide of either orientation (hsp7+BiP-OVA or hsp70+OVA-BiP) evoked a higher immune response as measured by specific lysis of cells than the hsp70 or TiterMax® adjuvant plus Ova-peptide alone.

#### EXAMPLE 4

The assay of Example 3 was repeated using CTL cell lines which had been maintained by stimulation with irradiated stimulators, syngeneic splenic feeder cells plus T cell growth factors for a period of two weeks. As shown in Fig. 2, the combination of hsp70 and the hybrid peptide of either orientation (hsp70+BiP-OVA or hsp7+OVA-BiP) evoked a higher immune response as measured by specific lysis of cells than the hsp70 or TiterMax® adjuvant plus Ova-peptide alone. Thus, the immune response elicited by the hybrid peptides persisted through additional passages and can be maintained over a period of time.

#### EXAMPLE 5

The experiment of Example 2 was repeated for the combinations of hsp70 plus BiP-OVA and TiterMax® plus Ova-peptide using only a single immunization one week before removal of the spleen cells. As shown in Fig. 3, the single immunization with either composition was effective in eliciting a cellular immune response.

#### EXAMPLE 6

The assay of Example 3 was repeated using mixtures of TiterMax® with Ova-peptide or the hybrid peptides of Example 1. As shown in Fig. 4, no significant difference was observed between the Ova-peptide and hybrid peptides demonstrating the specificity of the effect when hybrid peptides are used in association with the heat shock protein.

EXAMPLE 7

Figs. 5A and 5B show the results when the procedure of Example 3 was repeated immunizing the mice with hsp70 alone, OVA-peptide alone, Ova-BiP alone or Bip-Ova alone. As shown, the results in all cases were the same when the cells were pulsed with Ova-peptide (Fig. 5A) and when they had not been pulsed. (Fig. 5B). This demonstrates that the response is the result of the combination of the mixture of the antigen (Ova-peptide or hybrid peptide) and the heat shock protein and not to any of the components individually.

EXAMPLE 8

$^{14}\text{C}$ -labeled OVA-BiP was prepared by alkylation of OVA-BiP with  $^{14}\text{C}$ -formaldehyde. 0.9 mg of OVA-BiP in 300  $\mu\text{L}$  10% DMSO/water was added to 175  $\mu\text{L}$  of  $^{14}\text{C}$ -formaldehyde (62  $\mu\text{Ci}$ ) and immediately 50  $\mu\text{L}$  of freshly made up 200 mM NaCNBH<sub>3</sub> was added. The reaction was mixed and left at 25°C for 3 hours. The labeled peptide was repurified by reverse phase HPLC on a C-4 column in a 15 minute 0-100% acetonitrile (0.1% TFA) gradient.

The ability of the OVA-BiP peptide to bind to heat shock proteins was measured by incubating 100  $\mu\text{M}$  (5 $\mu\text{g}$ )  $^{14}\text{C}$ -labeled OVA-BiP with 50  $\mu\text{g}$  of BiP (prepared as in example 11), hsp70 (as prepared in Example 2) or grp96 (prepared as in Example 10) in a final volume of 20  $\mu\text{L}$  of buffer (50 mM Mops, pH 7.2., 200 mM NaCl, 5 mM MgAcetate) at 37°C for 30 minutes. The samples were then spun down (5 minutes in a microfuge) and loaded onto a 17 cm long Sephacryl S-300 column equilibrated in binding buffer (50 mM Mops, pH 7.2., 200 mM NaCl, 5 mM MgAcetate) and fractions were collected dropwise. 50  $\mu\text{L}$  of each ~225  $\mu\text{L}$  fraction was counted in scintillation liquid. 10  $\mu\text{L}$  of each fraction was also run on a 12% SDS-PAGE reducing gel. Fig. 6 shows the radioactivity detected in each fraction eluted from the column, together with the center of the peak of heat shock protein as determined by SDS-PAGE. As shown, a significant amount of radioactivity elutes with BiP and hsp70, thus providing

evidence that the hybrid peptide binds to these two heat shock proteins. The result for gp96 is unclear because the peak at fraction 11 (which may represent an aggregation phenomenon) and the gp96 peak (fraction 14) elute close together on the column used.

#### EXAMPLE 9

To prepare  $^{125}\text{I}$ -OVA-BiP, 250  $\mu\text{Ci}$  of monoiodinated Bolton-Hunter reagent was transferred into a stoppered vial and the solvent in which it was dissolved was evaporated with a gentle stream of argon gas. To the dried reagent 222  $\mu\text{L}$  of 4.5 mg/mL OVA-BiP in 100 mM  $\text{NaBO}_3$ , pH 8, 9, 10% DMSO was added. The reaction was mixed and incubated at  $25^\circ\text{C}$  for 45 minutes and continued at  $4^\circ\text{C}$  for a further hour. The labeled peptide was repurified by reverse phase HPLC on a C-4 column in a 20 minute, 0-100% acetonitrile (0.1% TFA) gradient.

The iodinated OVA-BiP was combined with BiP in substantially the same manner as the heat shock proteins in Example 7, except that since the iodinated peptide was at a very low concentration, 1  $\mu\text{L}$  (approx 32 ng) of labeled peptide was mixed with 5  $\mu\text{g}$  of unlabeled peptide and this was incubated with 50  $\mu\text{g}$  of BiP in 20  $\mu\text{L}$  of binding buffer. To observe ATP-mediated peptide release, ATP was added to a final concentration of 2 mM after the 30 minute incubation and incubated for a further 5 minutes prior to spinning. These samples were run on the same column as above, but equilibrated in binding buffer supplemented with 2 mM ATP.

Fig. 7 shows the elution profile for a mixture of the  $^{125}\text{I}$ -OVA-BiP and BiP in the presence and absence of 2 mM ATP. As shown, addition of ATP causes the release of the hybrid peptide from the BiP. This is consistent with the observation that ATP mediates release of bound proteins or polypeptides from heat shock proteins.

#### EXAMPLE 10



Hybrid peptides for use in a vaccine in accordance with the invention against human papilloma virus are prepared using a peptide synthesizer as follows:

E7 (Type 11) - BiP

- 5 Leu-Leu-Leu-Gly-Thr-Leu-Asn-Ile-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 10]

BiP-E7 (Type 11)

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Leu-Leu-Leu-Gly-Thr-Leu-Asn-Ile-Val [SEQ ID No. 11]

- 10 E7 (Type 16) - BiP

Leu-Leu-Met-Gly-Thr-Leu-Gly-Ile-Val-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 12]

BiP-E7 (Type 16)

- 15 His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Leu-Leu-Met-Gly-Thr-Leu-Gly-Ile-Val [SEQ ID No. 13]

E7 (Type 18) - BiP

Thr-Leu-Gln-Asp-Ile-Val-Leu-His-Leu-gly-ser-gly-His-Trp-Asp-Phe-Ala-Trp-Pro-Trp [SEQ ID No. 14]

BiP-E7 (Type 18)

- 20 His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Thr-Leu-Gln-Asp-Ile-Val-Leu-His-Leu [SEQ ID No. 15]

E7.1 (Type 6b)-BiP

Gly-Leu-His-Cys-Tyr-Glu-Gln-Leu-Val-gly-ser-gly-His-Trp-Asp-  
Phe-Ala-Trp-Pro-Trp [SEQ ID No. 16]

BiP-E7.1 (Type 6b)

5 His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Gly-Leu-His-Cys-  
Tyr-Glu-Gln-Leu-Val [SEQ ID No. 17]

E7.2 (Type 6b)-BiP

Pro-Leu-Lys-Gln-His-Phe-Gln-Ile-Val-gly-ser-gly-His-Trp-Asp-  
Phe-Ala-Trp-Pro-Trp [SEQ ID No. 18]

10 BiP-E7.2 (Type 6b)

His-Trp-Asp-Phe-Ala-Trp-Pro-Trp-gly-ser-gly-Pro-Leu-Lys-Gln-  
His-Phe-Gln-Ile-Val [SEQ ID No. 19]

Hybrid polypeptides for use in vaccines against  
human papilloma virus of other types or proteins from other  
15 viruses, bacteria etc can be developed by searching their  
sequences for MHC class I restricted peptide epitopes con-  
taining the HLA-A2 peptide binding motif.

EXAMPLE 11PREPARATION OF RECOMBINANT GP96

20 The DNA sequence encoding a wild-type or KDEL-  
deleted gp96 polypeptide was subcloned from pRc/CMV into the  
vector pET11a (Novagen). Thus upon expression, mature gp96  
could be purified from cell lysates.

Vector construction

25 PCR amplification of the sequence encoding gp96  
(from pRc/CMV) was performed with the following primers.  
The 5' primer for both wild-type and KDEL-deleted gp96 was  
complementary to the DNA sequence encoding the amino  
terminal end of the mature form of gp96 and an Nde I  
30 restriction site (CATATG) the ATG of which forms the  
initiator codon:

5' AGA TAT ACA TAT GGA TGA TGA AGT CGA CGT GG 3'

[SEQ ID No. 20]

The 3' primers were complementary to the DNA sequence of gp96 encoding the carboxyl terminal end of the protein, with the nucleotides encoding the KDEL sequence removed in the primer for the KDEL-deleted variant. Both primers contain a BamH I restriction site (GGATCC) followed by a STOP codon as shown: Wild-type:

5' TCG GAT CCT TAC AAT TCA TCC TTC TCT GTA GAT TC 3'

[SEQ ID No. 21]

KDEL-deleted:

5' TCG GAT CCT TAC TCT GTA GAT TCC TTT TC 3'

[SEQ ID No. 22]

The PCR products were cut with Nde I and BamH I and ligated into pET11a (Novagen) which had also been cut with these enzymes. The ligation product was used to transform competent BL21 cells. Clones obtained were screened by expression screening.

#### Expression and Purification

This procedure is identical for wild-type or KDEL-deleted gp96. Two liters of *E. coli* BL21 cells transformed with pET11a containing a sequence coding for either wild-type or KDEL-deleted gp96 were grown in 2xTY medium supplemented with 200 ug/ml ampicillin at 37°C until they reached an absorbance at 600 nm of 0.5-0.6 at which point they were induced by the addition of 1 mM IPTG. The cells were allowed to grow for a further 2-5 hours at 37°C and then they were harvested by 10 minutes centrifugation at 7000 x G. The cell pellet was resuspended in 50mM Hepes pH 7.5, 50mM KCl, 5mM MgAcetate, 20% sucrose, 1mM PMSF and the cells lysed by passing them through the French Press three times. The cell extract was clarified by a one hour spin at 200000 x G and the supernatant retained.

The supernatant was diluted two-fold with cold 50 mM Hepes pH 7.5 and loaded onto a Pharmacia XK26 column containing 50 ml of DE52 anion exchange resin (Whatman) which

had been equilibrated in 50 mM Mops pH 7.4., 10 mM NaCl, 5 mM MgAcetate. The bound protein was eluted in a 0-1000 mM NaCl gradient. Fractions containing gp96 were identified by SDS-PAGE and pooled.

5           The pooled gp96-containing fractions were diluted two-fold with cold 50 mM Mops pH 7.4 and loaded onto a Pharmacia XK16 column containing 15 mL of hydroxylapatite resin (BioRad) which had been washed with 0.5 M  $K_2HPO_4$  pH 7.2., 50 mM KCl and equilibrated in 10 mM  $K_2HPO_4$  pH 7.2, 50mM KCl.  
10       The bound protein was eluted in a 10-500 mM  $K_2HPO_4$  pH 7.2 gradient with the KCl concentration held constant at 50mM. Fractions containing gp96 were identified by SDS-PAGE and pooled.

15           The pooled gp96-containing fractions were finally loaded onto a Pharmacia XK26 column containing 25ml of phenyl Sepharose (Pharmacia) which had been equilibrated in 50mM Mops pH 7.2, 500mM NaCl and eluted in a 500-0mM NaCl gradient. The fractions containing essentially pure gp96 were pooled, concentrated by filtration and made up to 10%  
20       glycerol. The purified gp96 was stored frozen at -80°C.

#### EXAMPLE 12

##### Construction of BiP Expression vector and Purification of Recombinant BiP

25       The DNA sequence encoding the wild-type or KDEL-deleted BiP polypeptide was subcloned from pCDNA3 into the vector pET22 (Novagen), thereby placing it behind and in frame with a DNA sequence that codes for a signal sequence which targets the expressed BiP to the periplasmic space of the bacterial expression host, *E. coli*. Upon transport into  
30       the periplasm the signal sequence is removed and thus mature wild-type or KDEL-deleted BiP can be harvested from the periplasm without any contamination by cytosolic hsp70s.

##### Vector construction:

35       PCR amplification of the sequence encoding BiP (from pCDNA3) was performed with the following primers. The 5'

primer for both wild-type and KDEL-deleted BiP was complementary to the DNA sequence of BiP encoding the amino terminal end of the mature form of BiP with an Msc I restriction site (TGGCCA) immediately upstream from the initiator ATG codon:

5' AGA TAT GTG GCC ATG GAG GAG GAG GAC AAG 3'

[SEQ ID No. 23]

The 3' primers were complementary to the DNA sequence of BiP encoding the carboxyl terminal end of the protein, with the nucleotides encoding the KDEL sequence removed in the primer for the KDEL-deleted variant. Both primers contain a BamH I restriction site (GGATCC) followed by stop codon as shown:

Wild-type:

5' TCG GAT CCC TAC AAC TCA TCT TTT TCT G 3'

[SEQ ID No. 24]

KDEL-deleted:

5' TCG GAT CCC TAT TCT GAT GTA TCC TCT TCA CC 3'

[SEQ ID No. 25]

The PCR products were cut with Msc I and BamH I and ligated into pET22 (Novagen) which had also been cut with these enzymes. The ligation product was used to transform competent BL21 cells. Clones obtained were screened by expression screening.

#### Expression and Purification

The procedure is identical for wild-type or KDEL-deleted BiP. Two liters of BL21 cells transformed with pET22 containing a sequence coding for either wild-type or KDEL deleted BiP were grown in 2xTY medium supplemented with 200µg/ml ampicillin at 37°C until they reached an absorbance at 600 nm of 0.5-0.6 at which point they were induced by the addition of 1 mM IPTG. The cells were allowed to grow for a further 2-5 hours at 37°C and then they were harvested by 10 minutes centrifugation at 7000 x G. The cell pellet was gently resuspended in 400 mL (or 80 mL/gm cells ) of 30 mM Tris pH 8.0, 20% Sucrose, 1 mM PMSF. Following resuspension of the cells EDTA was added to 1 mM and the suspension

-28-

incubated at room temperature for 5 minutes. The cells were then spun down for 15 minutes at 7000 x G and resuspended in 400 mL of ice cold 5 mM MgSO<sub>4</sub>, 1 mM PMSF and incubated at 4°C for 10 minutes. The cells were then spun down once again and the supernatant kept since this now constitutes the periplasmic extract.

The periplasmic extract was loaded onto a Pharmacia XK26 column containing 25 mL of DE52 anion exchange resin (Whatman) which had been equilibrated in 50 mM Mops pH 7.4, 10 mM NaCl. The bound protein was eluted in a 10-500 mM NaCl gradient. Fractions containing eluted BiP were identified by SDS-PAGE and pooled. The pooled BiP was subsequently run onto a Pharmacia XK26 column containing 10 mL of ATP agarose which had been equilibrated in 50 mM Mops pH 7.4., 100 mM NaCl, 5 mM MgAcetate, 10 mM KCl. After loading the pooled BiP containing fractions the column was washed until the baseline of absorption at 280 nm reached zero. Finally the bound BiP was eluted with the same buffer supplemented with 1 mM ATP. The eluate was concentrated by filtration, made up to 10% glycerol and stored frozen at -80°C.

### EXAMPLE 13

#### PREPARATION OF RECOMBINANT MOUSE HSP40

##### Plasmid Constructions

The DNA fragment used to introduce an Nde I site at the initiation methionine of hsp40 was constructed via polymerase chain reaction (PCR) using an Nde-primer 5'-CCGCAGGAGGGGCATATGGGTAAAGAC-3' [SEQ ID No. 26] and an Nco-primer 5'-GAGGGTCTCCATGGAATGTGTAGCTG-3' [SEQ ID No. 27]. The latter included an Nco I site corresponding to nucleotide 322 of the human hsp40 cDNA clone, pBSII-hsp40, Ohtsuka, K., *Biochem. Biophys. Res. Commun.* 197: 235-240 (1991), which was used as the template. The Hsp40-coding region of pBSII-hsp40 was digested with BamH I and Sac I and inserted into the complementary sites in a modified form of the plasmid pET-3a (Novagen, Inc.). The PCR-amplified DNA

was digested with Nde I and Nco I, and replaced the Nde I-Nco I region of the above plasmid to create the plasmid pET/hsp40, expressing hsp40.

Protein Purification:

5 To purify recombinant human hsp40, the plasmid pET/hsp40 was transformed into BL21(DE3) cells grown at 37°C. After a 2 hour incubation with 0.4 mM isopropyl thio-b-D-galactoside (IPTG), cells were lysed in a French Pressure Cell (SLM Instruments, Inc.) in buffer A [20 mM  
10 Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA] containing 1 mM PMSF. The cleared lysate was mixed with DEAE-Sephacel (Pharmacia) on ice for 1 h. The unbound material was collected and the resin was washed with buffer A. The flow-through and first wash were combined and loaded onto a  
15 hydroxyapatite HTP column (Bio-Rad) equilibrated with 100 mM potassium phosphate, pH 7.6. The column was washed with the same buffer and Hsp40 was eluted with a linear gradient of 100-300 mM potassium phosphate, pH 7.6. Peak fractions were rechromatographed on an HTP column after passing them  
20 through a DEAE-Sephacel column.

EXAMPLE 14

Vaccine compositions were prepared by combining recombinant mouse hsp70 (prepared as in example 2), recombinant human hsp40 (prepared as in example 13) and Ova-peptide  
25

Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu [SEQ ID NO. 7]  
in a final volume of 10 µl of buffer (20 mM Hepes pH 7.0, 150 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgCl<sub>2</sub> and 2 mM MgATP) as follows:

30

Sample	hsp70	hsp40	ova
OVA-alone	nil	nil	5 ug
Hsp70/40	15 ug	8 ug	nil

Hsp70/40 + OVA	15 ug	8 ug	5 ug
Hsp70 + OVA	15 ug		5 ug

The mixtures were incubated for 30 minutes at 37°C prior to use for immunizations.

C57BL/6 mice were immunized intradermally once a week for a total of two weeks with 10 uL of one of the mixtures described above or with a mixture of TiterMax® (Vaxcell, Norcross, GA) and Ova-peptide (5 ug). One week after the second immunization, spleen cells were removed and mononuclear cells ( $6-8 \times 10^7$ ) were cultured with  $3 \times 10^6$   $\gamma$ -irradiated (3000 rad) stimulator cells. The stimulator cells were obtained from naive mice that had been sensitized in vitro with ova peptide (10 mg/ml) for 30 minutes at room temperature, washed and irradiated at 3000 rads.

Cytotoxicity of spleen cells from vaccinated mice was assayed on Ova-peptide pulsed EL4 cells in an 18-hour chromium release assay. CTL were generated by culturing in vivo immunized spleen cells for 5 days at a concentration of  $10^6$  cells/mL in RPMI medium, 10% FCS, penicillin, streptomycin and 2 mM L-glutamine, together with  $3 \times 10^6$   $\gamma$ -irradiated (3,000 rad) stimulator cells/mL. Target cells were prepared by culturing cells for 1 hour in the presence of 250 uCi of  $^{51}\text{Cr}$  sodium chromate (DuPont, Boston, MA) in Tris-phosphate buffer, pH 7.4 at 37°C for 60 minutes. After washing,  $10^4$   $^{51}\text{Cr}$ -labeled target cells were mixed with effector lymphocytes to yield several different effector/target (E/T) ratio and were incubated for 18 hours. Supernatants were harvested and the radioactivity was measured in a gamma counter. Percent specific lysis was calculated as:  $100 \times [\text{Cpm release by CTL} - \text{cpm spontaneous release}] / (\text{cpm maximal release} - \text{cpm spontaneous release})$ . Maximal response was determined by addition of 1% Triton X-100. Spontaneous release of all target in the absence of effector cells was less than 25% of the maximal release.



The results of this study are shown in Fig. 8. As shown, combinations of antigen with hsp70 or a mixture of hsp70 and hsp40 are effective to produce a CTL response to the antigen, while the administration of the antigen alone or a combination of heat shock proteins is not.

#### EXAMPLE 15

The experiment of Example 14 was repeated using EG7 lymphoma cells, Moore et al., Cell 54: 777-785 (1988), in place of the EL4 cells. The results are shown in Fig. 9 and are comparable to those observed using EL4 cells.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entireties.

- 32 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Sloan-Kettering Institute for Cancer Research

ROTHMAN, James E.  
HARTL, F. Ulrich  
HOE, Mee H.  
HOUGHTON, Alan  
TAKEUCHI, Yoshizumi  
MAYHEW, Mark

(ii) TITLE OF INVENTION: Method for Treatment of Cancer and Infectious Diseases and Compositions Useful in Same

(iii) NUMBER OF SEQUENCES: 28

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Oppedahl & Larson  
(B) STREET: 1992 Commerce Street Suite 309  
(C) CITY: Yorktown  
(D) STATE: NY  
(E) COUNTRY: US  
(F) ZIP: 10598

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette - 3.5 inch, 1.44 Mb storage  
(B) COMPUTER: IBM compatible  
(C) OPERATING SYSTEM: MS DOS  
(D) SOFTWARE: Word Perfect

(vi) CURRENT APPLICATION DATA :

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION :

(A) NAME: Larson, Marina T.  
(B) REGISTRATION NUMBER: 32,038  
(C) REFERENCE/DOCKET NUMBER: MSK.P-019-WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (914) 245-3252  
(B) TELEFAX: (914) 962-4330  
(C) TELEX:

## (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: mouse  
(ix) feature

-33-

(D) OTHER INFORMATION: heat shock binding sequence  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
His Trp Asp Phe Ala Trp Pro Trp

5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) feature

(D) OTHER INFORMATION: HLA-A2 peptide binding motif, Leu at position 2 may also be Met, Val at position 6 may also be Ile, Leu or Thr; Val at position 9 may also be Leu

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Leu Xaa Xaa Xaa Val Xaa Xaa Val

5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) feature

(D) OTHER INFORMATION: HLA-A2 peptide binding motif, Leu at position 2 may also be Met

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Xaa Leu Xaa Xaa Xaa Xaa Xaa Val

5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- 34 -

- (A) ORGANISM:
- (ix) feature
- (D) OTHER INFORMATION: heat shock protein binding motif
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Phe Trp Gly Leu Trp Pro Trp Glu  
5

- (2) INFORMATION FOR SEQ ID NO: 5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 5
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (iii) HYPOTHETICAL: no
  - (v) FRAGMENT TYPE: internal
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM: human
    - (ix) feature
  - (D) OTHER INFORMATION: HLA-DR peptide binding motif
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gln Lys Arg Ala Ala  
5

- (2) INFORMATION FOR SEQ ID NO: 6:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 5
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (iii) HYPOTHETICAL: no
  - (v) FRAGMENT TYPE: internal
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM: human
    - (ix) feature
  - (D) OTHER INFORMATION: HLA-DR peptide binding motif
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gln Lys Arg Ala Ala  
5

- (2) INFORMATION FOR SEQ ID NO: 7:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 8
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (iii) HYPOTHETICAL: no

-35-

- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: chicken
  - (ix) feature
- (D) OTHER INFORMATION: OVA-peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Ile Ile Asn Phe Glu Lys Leu  
5

- (2) INFORMATION FOR SEQ ID NO: 8:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (iii) HYPOTHETICAL: no
  - (v) FRAGMENT TYPE: internal
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM:
    - (ix) feature
  - (D) OTHER INFORMATION:
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Ile Ile Asn Phe Glu Lys Leu Gly Ser Gly His  
5 10

Trp Asp Phe Ala Trp Pro Trp  
15

- (2) INFORMATION FOR SEQ ID NO: 9:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19
    - (B) TYPE: amino acid
    - (C) STRANDEDNESS:
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
  - (iii) HYPOTHETICAL: no
  - (v) FRAGMENT TYPE: internal
  - (vi) ORIGINAL SOURCE:
    - (A) ORGANISM:
    - (ix) feature
  - (D) OTHER INFORMATION:
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

His Trp Asp Phe Ala Trp Pro Trp Gly Ser Gly Ser  
5 10

Ile Ile Asn Phe Glu Lys Leu  
15

-36-

## (2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL:yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION:hybrid peptide for human papilloma virus vaccine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu	Leu	Leu	Gly	Thr	Leu	Asn	Ile	Val	Gly	Ser	Gly
				5					10		
His	Trp	Asp	Phe	Ala	Trp	Pro	Trp				
		15					20				

## (2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL:yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION:hybrid peptide for human papilloma virus vaccine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

His	Trp	Asp	Phe	Ala	Trp	Pro	Trp	Gly	Ser	Gly	Leu
				5					10		
Leu	Leu	Gly	Thr	Leu	Asn	Ile	Val				
		15					20				

## (2) INFORMATION FOR SEQ ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL:yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

Leu Leu Met Gly Thr Leu Gly Ile Val Gly Ser Gly  
5 10  
His Trp Asp Phe Ala Trp Pro Trp  
15 20

(D) OTHER INFORMATION:hybrid peptide for human papilloma virus vaccine

His Trp Asp Phe Ala Trp Pro Trp Gly Ser Gly Leu  
5 10  
Leu Met Gly Thr Leu Gly Ile Val  
15 20

(D) OTHER INFORMATION:hybrid peptide for human papilloma virus vaccine

Thr Leu Gln Asp Ile Val Leu His Leu Gly Ser Gly  
5 10  
His Trp Asp Phe Ala Trp Pro Trp  
15 20

- His Trp Asp Phe Ala Trp Pro Trp Gly Ser Gly Thr  
5 10  
Leu Gln Asp Ile Val Leu His Leu  
15 20

- (2) INFORMATION FOR SEO ID NO: 16:

- ```

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: yes
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(ix) feature
(D) OTHER INFORMATION: hybrid peptide for human papilloma
virus vaccine
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Gly Leu His Cys Tyr Glu Gln Leu Val Gly Ser Gly  
5 10  
His Trp Asp Phe Ala Trp Pro Trp  
15 20

- (2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTIC:  
(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: peptide  
(iii) HYPOTHETICAL: yes



```
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(ix) feature
(D) OTHER INFORMATION:hybrid peptide for human papilloma
virus vaccine
(xi) SEQUENCE DESCRIPTION: SEO ID NO:17:
```

His Trp Asp Phe Ala Trp Pro Trp Gly Ser Gly Gly.  
5 10  
Leu His Cys Tyr Glu Gln Leu Val  
15 20

```
(2) INFORMATION FOR SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: yes
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM:
(ix) feature
(D) OTHER INFORMATION: hybrid peptide for human papilloma
virus vaccine
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
```

Pro Leu Lys Gln His Phe Gln Ile Val Gly Ser Gly  
5 10  
His Trp Asp Phe Ala Trp Pro Trp  
15 20

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: yes
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM:
  - (ix) feature
- (D) OTHER INFORMATION: hybrid peptide for human papilloma virus vaccine
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

His Trp Asp Phe Ala Trp Pro Trp Gly Ser Gly Pro

5 10

-40-

Leu Lys Gln His Phe Gln Ile Val  
15 20

## (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for gp96

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGATATACAT ATGGATGATG AAGTCGACGT GG 32

## (2) INFORMATION FOR SEQ ID NO: 21:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for gp96

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGGATCCTT ACAATTCATC CTTCTCTGTA GATTC 35

## (2) INFORMATION FOR SEQ ID NO: 22:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

-41-

- (D) OTHER INFORMATION: amplification primer for gp96  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCGGATCCTT ACTCTGTAGA TTCCTTTTC 29

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for BiP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGATATGTGG CCATGGAGGA GGAGGACAAG 30

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for BiP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCGGATCCCT ACAATCATC TTTTCTG 28

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

- 42 -

(ix) feature

(D) OTHER INFORMATION: amplification primer for BiP

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCGGATCCCT ATTCTGATGT ATCCTCTTCA CC

32

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: yes

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for hsp40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCGCAGGAGG GGCATATGGG TAAAGAC

27

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTISENSE: no

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

(ix) feature

(D) OTHER INFORMATION: amplification primer for hsp40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAGGGTCTCC ATGGAATGTG TAGCTG

26

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: no

(v) FRAGMENT TYPE: internal

-43-

(vi) ORIGINAL SOURCE:  
(A) ORGANISM:human  
(ix) feature  
(D) OTHER INFORMATION: HLA-A2 peptide binding motif, Val at  
position 2 may also be Gln  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:  
Xaa Val Xaa Xaa Xaa Xaa Xaa Xaa Leu

CLAIMS

1           1. A composition for inducing a therapeutic immune  
2 response in a subject, comprising:

3           (a) a target antigen; and

4           (b) a heat shock protein;

5           wherein the target antigen and the heat shock  
6 protein are combined *in vitro* under conditions wherein  
7 binding of target antigen to heat shock protein occurs to  
8 form a target antigen/heat shock protein complex;

9           wherein the administration of the target  
10 antigen/heat shock protein complex to the subject induces an  
11 immune response comprising a cytotoxic cellular component.

1           2. The composition of claim 1, wherein the heat  
2 shock protein is hsp70.

1           3. The composition of claim 1, wherein the heat  
2 shock protein is gp96.

1           4. The composition of claim 1, wherein the heat  
2 shock protein is hsp40.

1           5. The composition of claim 1, wherein the heat  
2 shock protein is BiP.

1           6. The composition of any of claims 1 to 5, wherein  
2 the target antigen is a hybrid antigen.

1           7. The composition according to claim 6 wherein the  
2 hybrid antigen comprises an antigenic domain derived from a  
3 first source and a binding domain which binds to a heat  
4 shock protein from a second source different from the first  
5 source.

1           8. The composition of claim 7, wherein the binding  
2 domain comprises at least a heptameric region having the  
3 sequence

4                           HyXHyXHyXHy

5 where Hy represents a hydrophobic amino acid residue and X  
6 is any amino acid.

1           9. The composition of claim 7, wherein the binding  
2 domain comprises a region having the sequence  
3 His Trp Asp Phe Ala Trp Pro Trp           [Seq. ID No. 1]

1           10. A composition for inducing a therapeutic immune  
2 response in a subject, comprising:

3           (a) a nucleic acid molecule comprising a region  
4 encoding a target antigen operably linked to a promoter  
5 element; and

6           (b) a nucleic acid molecule comprising a region  
7 encoding a heat shock protein operably linked to a promoter  
8 element;

9           wherein the introduction of the nucleic acids of (a)  
10 and (b) into a cell result in the binding of target antigen  
11 to heat shock protein.

1           11. The composition of claim 10, wherein the nucleic  
2 acid molecules of (a) and (b) are comprised in the same  
3 vector.

1           12. The composition of claim 10 or 11, wherein the  
2 heat shock protein is hsp70.

1           13. The composition of claim 10 or 11, wherein the  
2 heat shock protein is gp96.

1           14. The composition of claim 10 or 11, wherein the  
2 heat shock protein is hsp40.

1           15. The composition of claim 10 or 11, wherein the  
2   heat shock protein is BiP.

1           16. The composition of any of claims 10 to 15,  
2   wherein the target antigen is a hybrid antigen.

1           17. The composition according to claim 16, wherein  
2   the hybrid antigen comprises an antigenic domain derived  
3   from a first source and a binding domain which binds to a  
4   heat shock protein. from a second source different from the  
5   first source.

1           18. The composition of claim 17, wherein the binding  
2   domain comprises at least a heptameric region having the  
3   sequence

4                           HyXHyXHyXHy

5   where Hy represents a hydrophobic amino acid residue and X  
6   is any amino acid.

1           19. The composition of claim 17, wherein the  
2   binding domain comprises a region having the sequence  
3   His Trp Asp Phe Ala Trp Pro Trp           [Seq. ID No. 1]

1           20. A method of inducing an immune response in a  
2   subject in need of such treatment, comprising administering  
3   to the subject a therapeutically effective amount of the  
4   composition of any of claims 1 to 19.

1           21. A hybrid peptide comprising:  
2               (a) an antigenic domain derived from a first  
3   source; and  
4               (b) a binding domain which binds to a heat  
5   shock protein, said binding domain being derived from a  
6   second source different from the first source.



1           22. The hybrid peptide of claim 21, wherein the  
2 antigenic domain is derived from a virus, a parasite, a  
3 mycoplasma, a fungus or a bacterium.

1           23. The hybrid peptide of any of claims 21-22,  
2 wherein the antigenic domain elicits an immune response to a  
3 neoplastic disease.

1           24. The hybrid peptide of claim 23, wherein the  
2 neoplastic disease is selected from among a sarcoma, a  
3 lymphoma, a carcinoma, a leukemia and a melanoma.

1           25. The hybrid peptide of any of claims 21 to 24,  
2 wherein the binding domain comprises at least a heptameric  
3 region having the sequence

4                           HyXHyXHyXHy

5 where Hy represents a hydrophobic amino acid residue and X  
6 is any amino acid.

1           26. The hybrid peptide of any of claim 21 to 24,  
2 wherein the binding domain comprises a region having the  
3 sequence

4 His Trp Asp Phe Ala Trp Pro Trp                           [Seq. ID No. 1].

1           27. The hybrid peptide of any of claims 21 to 24,  
2 wherein the binding domain comprises at least a pentapeptide  
3 region selected from among

4 Gln Lys Arg Ala Ala                           [Seq. ID No. 5], and

5 Arg Arg Arg Ala Ala                           [Seq. ID No. 6].

1           28. A polynucleotide construct comprising:

2           (a) a region encoding a hybrid peptide  
3 comprising an antigenic domain derived from a first source;  
4 and a binding domain which binds to a heat shock protein  
5 said binding domain being derived from a second source  
6 different from the first source;

7 (b) a promoter effective to promote expression  
8 on the hybrid peptide in mammalian cells.

1 29. The polynucleotide construct of claim 28,  
2 wherein the antigenic domain is derived from a virus, a  
3 parasite, a mycoplasma, a fungus or a bacterium.

1 30. The polynucleotide construct of claim 28,  
2 wherein the antigenic domain elicits an immune response to a  
3 neoplastic disease.

1 31. The polynucleotide construct of claim 30,  
2 wherein the neoplastic disease is selected from among a  
3 sarcoma, a lymphoma, a carcinoma, a leukemia and a melanoma.

1 32. The polynucleotide construct of any of claims  
2 28 to 31, wherein the binding domain comprises at least a  
3 heptameric region having the sequence

4 HyXH<sub>2</sub>HyXH<sub>2</sub>HyXH<sub>2</sub>

5 where Hy represents a hydrophobic amino acid residue and X  
6 is any amino acid.

1 33. The polynucleotide construct of any of claims  
2 28 to 31, wherein the binding domain comprises a region  
3 having the sequence  
4 His Trp Asp Phe Ala Trp Pro Trp [Seq. ID No. 1].

1 34. The polynucleotide construct of any of claim s  
2 28 to 31, wherein the binding domain comprises at least a  
3 pentapeptide region selected from among  
4 Gln Lys Arg Ala Ala [Seq. ID No. 5], and  
5 Arg Arg Arg Ala Ala [Seq. ID No. 6].

1/5

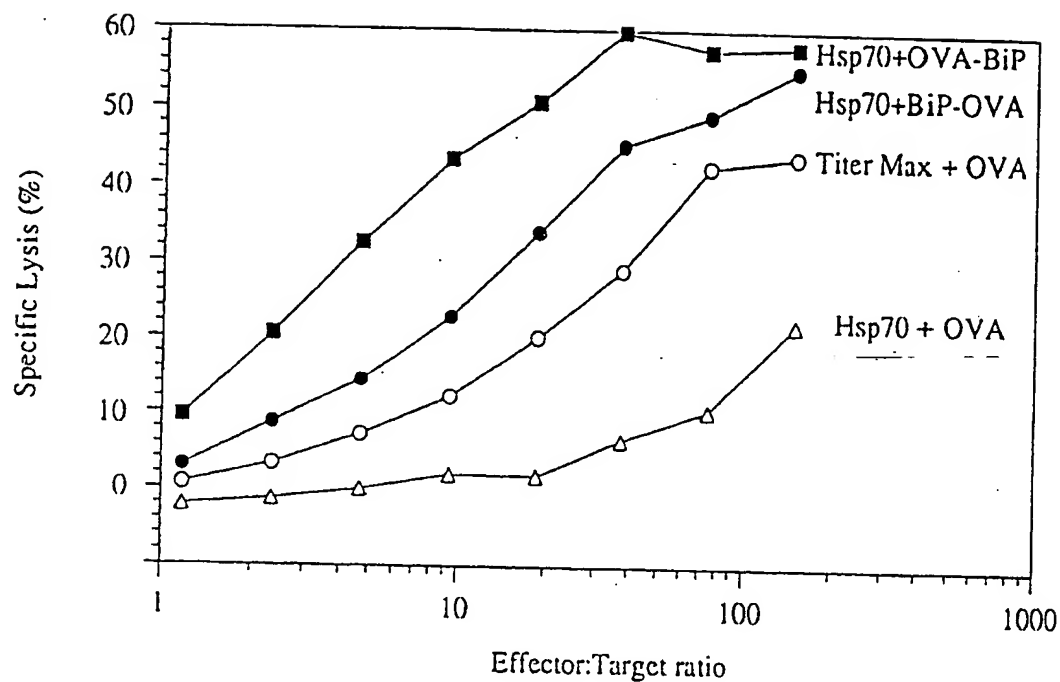


Fig. 1

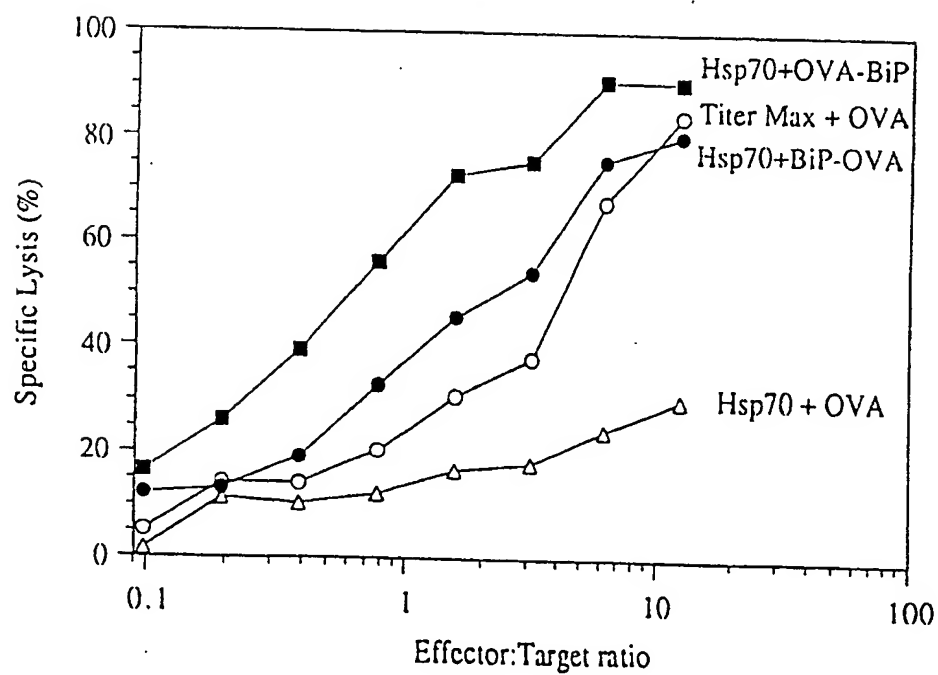


Fig. 2

2/5

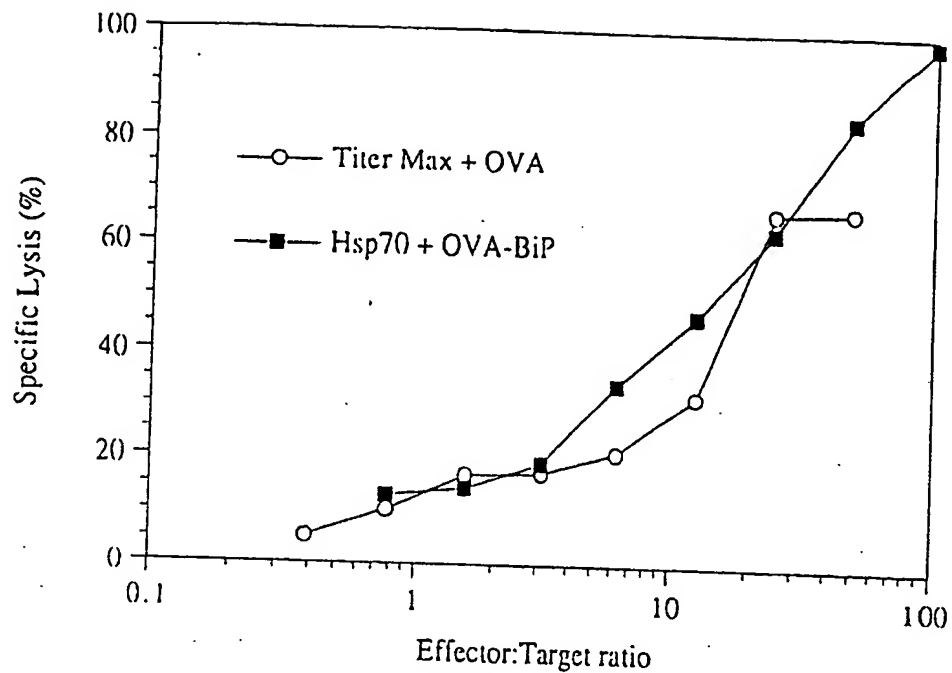


Fig. 3

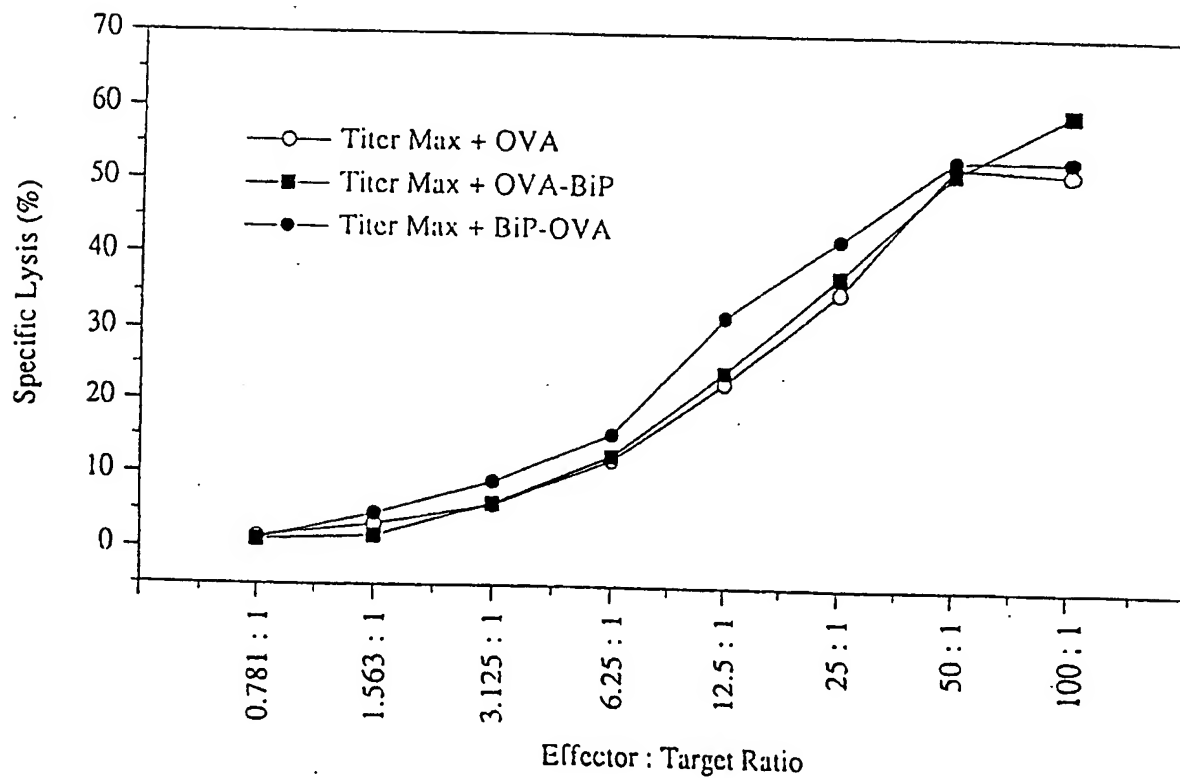


Fig. 4

3/5

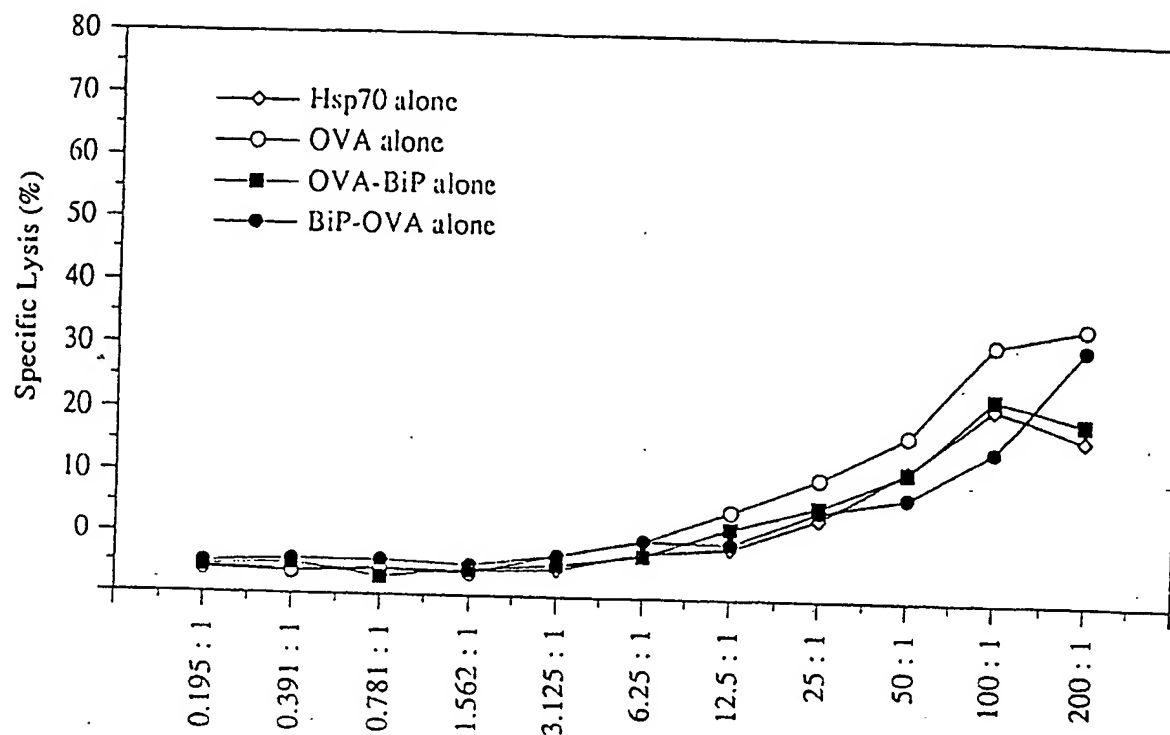


Fig. 5B

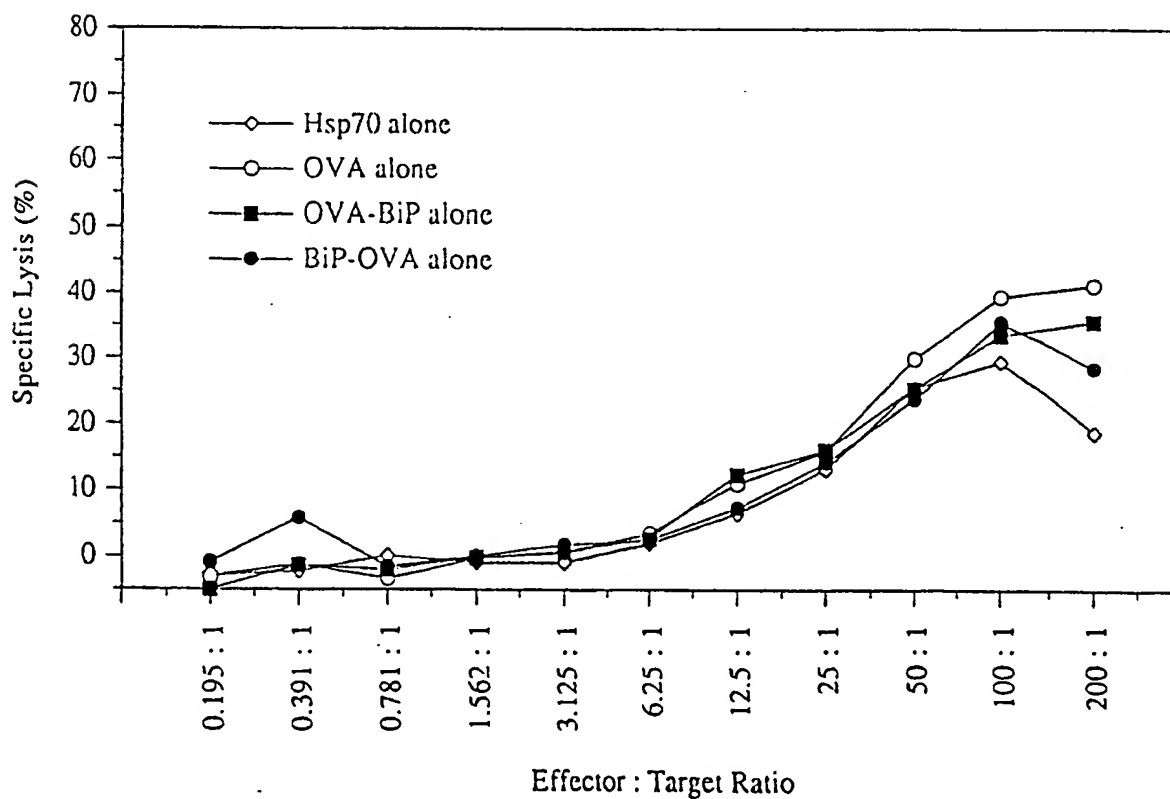


Fig. 5A

4/5

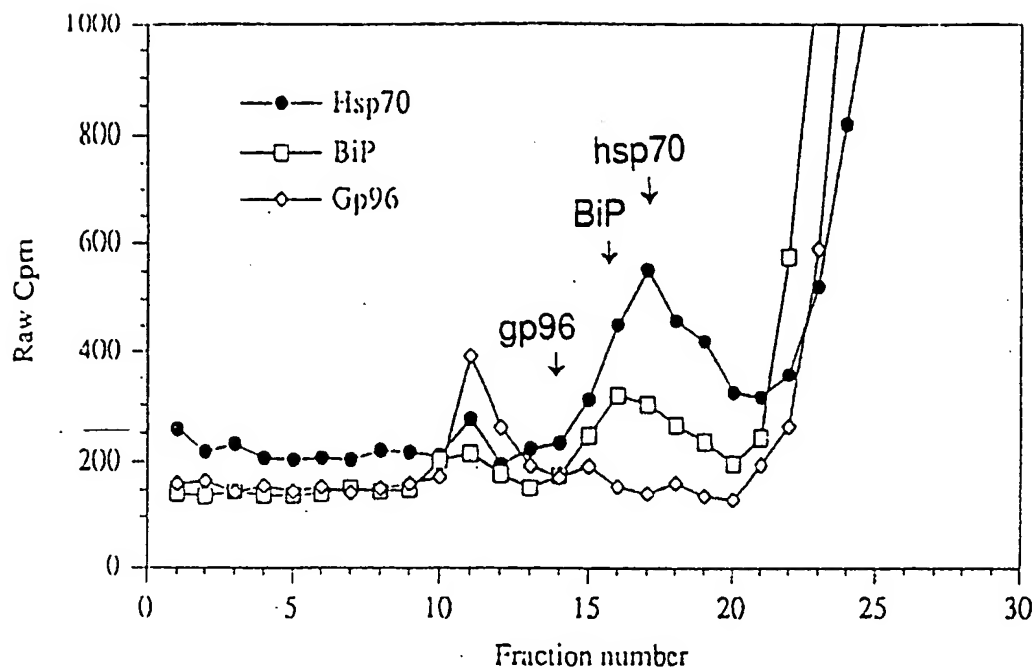


Fig. 6

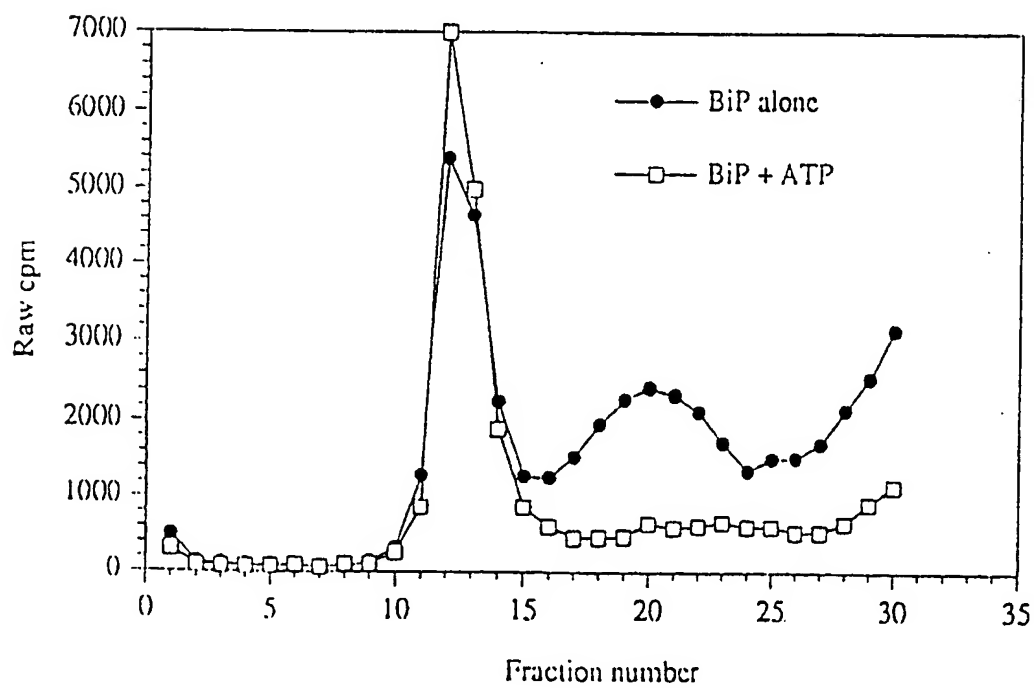


Fig. 7

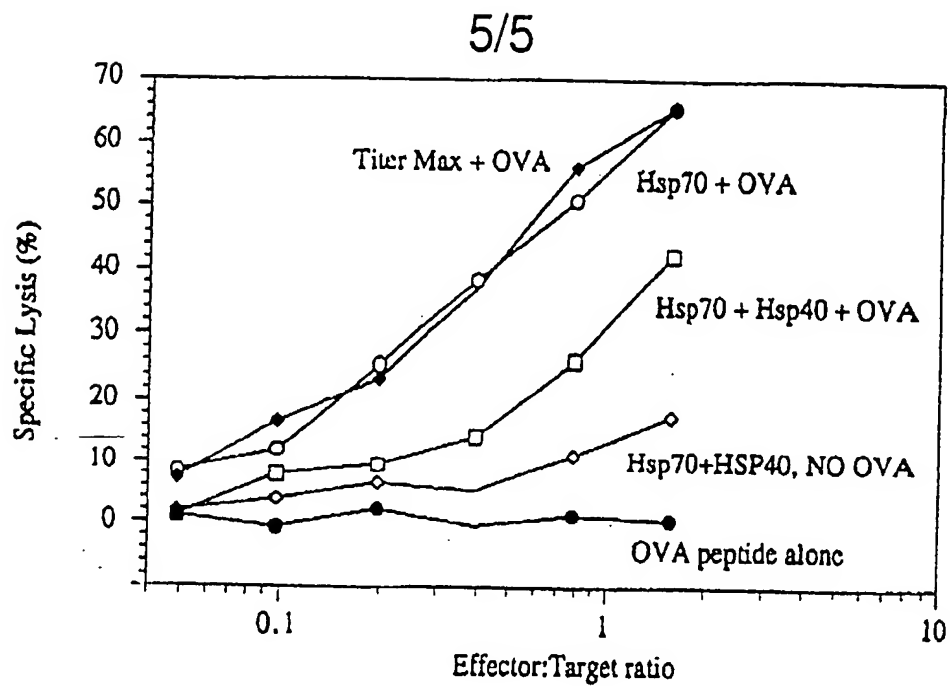


Fig. 8

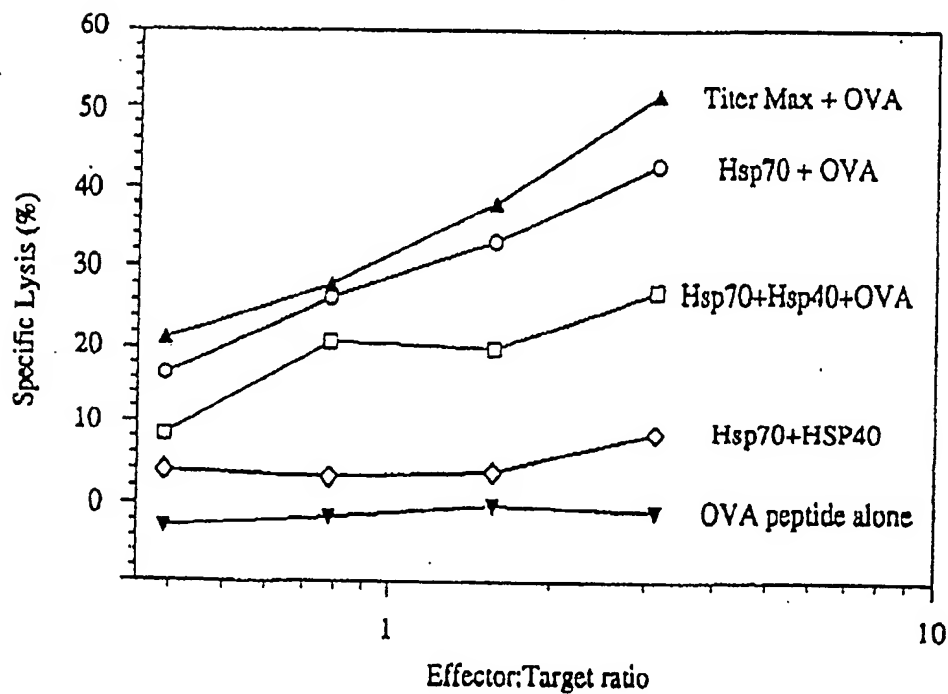


Fig. 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13363

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 39/00, 39/38; C12P 21/04; C07H 21/02, 21/04

US CL : 424/184.1, 192.1; 435/69.7; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 192.1; 435/69.7; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                   | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | DENAGEL et al. Heat Shock Proteins in Immune Responses. Critical Reviews in Immunology. 1993, Vol. 13, No. 1, pages 71-81, see entire document.                                                                      | 1-15, 21-24, 28-34    |
| Y         | RETZLAFF et al. Bacterial Heat Shock Proteins Directly Induce Cytokine mRNA and Interleukin-1 Secretion in Macrophage Cultures. Infection and Immunity. 1994, Vol. 62, No. 12, pages 5689-5693, see entire document. | 1-15, 21-24, 28-34    |

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                                | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| *A* document defining the general state of the art which is not considered to be of particular relevance                                                                | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| *E* earlier document published on or after the international filing date                                                                                                | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Z* document member of the same patent family                                                                                                                                                                                                    |
| *O* document referring to an oral disclosure, use, exhibition or other means                                                                                            |                                                                                                                                                                                                                                                  |
| *P* document published prior to the international filing date but later than the priority date claimed                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search

14 OCTOBER 1996

Date of mailing of the international search report

13 NOV 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

HEATHER BAKALYAR

Telephone No. (703) 308-0196



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13363

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                  | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | MUSTAFA et al. Human T Cells Recognize Mycobacterial Heat Shock Proteins in the Context of Multiple HLA-DR Molecules: Studies with Healthy Subjects Vaccinated with Mycobacterium Bovis BCG and Mycobacterium leprae. Infection and Immunity. December 1993, Vol. 61, No. 12, pages 5294-5301, see entire document. | 1-15, 21-24, 28-34    |
| Y         | MULTHOFF et al. A stress-inducible 72-kDa Heat-Shock Protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. Int. J. Cancer.— 1995, Vol. 61, pages 272-279, see entire document.                                                                                                 | 1-15, 21-24, 28-34    |
| Y         | YAMAMOTO et al. Listeria monocytogenes-induced gamma interferon secretion by intestinal intraepithelial gamma/delta T lymphocytes. Infection and Immunity. May 1993, Vol. 16, No. 5, pages 2154-2181, see entire document.                                                                                          | 1-15, 21-24, 28-34    |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13363

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☒ Claims Nos.: 16-19, 20, 25-27  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/13363

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG author and word: (heat shock protein, hsp, antigen, immunogen, immune, immunity, modulate?, increase? effect, target, neoplas?, gene(w)therapy, etc. )

medline, biosis, scisearch, embase, wpi, uspatful